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Induction of fusion-competent myoblast-specific gene expression during myogenic differentiation of *Drosophila* Schneider cells by DNA double-strand breaks or replication inhibition

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Abstract

Differentiation of *Drosophila* Schneider cells caused by DNA double-strand break (DSB)-inducing topoisomerase II (topo II) inhibitors were attenuated by ICRF-193, a non-DNA-damaging topo II inhibitor. ICRF-193 did not inhibit differentiation induced by neocarzinostatin (NCS), a drug that causes DNA DSBs independent of topo II. Schneider cells differentiated upon treatment with γ-ray. These results suggest that DNA DSBs induce myogenic differentiation of Schneider cells. We also found DNA replication inhibitors, hydroxyurea (HU), aphidicolin, and ethylmethanesulfonate (EMS) induced myogenic differentiation of Schneider cells. HU-induced differentiation was inhibited upon pretreatment of cells with chemical inhibitors of PP 1/2A, p38 MAPK, JNK, and proteasome. RT-PCR analysis revealed that the expressions of fusion-competent myoblast-specific genes *lmd*, *sns*, and *del* were induced in Schneider cells upon treatment with NCS or HU, whereas expressions of three founder cell-specific genes, *duf*, *ants*, and *rols*, were undetectable. These results indicate that the expression of fusion competent-myoblast-specific genes is induced during myogenic differentiation of *Drosophila* Schneider cells by DNA DSBs or replication inhibition.

Keywords: *Drosophila*; Myogenesis; Fusion-competent myoblast; Double-strand break; Replication inhibition

1. Introduction

Studies in *Drosophila* have provided invaluable information on many aspects of the molecular genetics and cell biology of muscle development. In *Drosophila*, somatic myogenesis begins soon after inception of the mesoderm in the early embryo. After subdivision of the mesoderm, specific muscle progenitor cells segregate, providing the embryo with a complete set of individual muscle founders [1,2]. Founder cells (FCs) first form bi- or trinucleated cells by fusion to the second class of mesodermal cells, the fusion-competent myoblasts (FCMs), and then enlarge by further fusion to form mature myotubes [3]. Fusion is asymmetric, that is, FCs fuse with FCMs, but neither myoblast type fuses with itself [4,5]. *Dumbfounded* (*duf*) and *sticks and stones* (*sns*) are expressed exclusively in the FCs and FCMs, respectively [6,7]. Both genes encode immunoglobulin superfamily members with the structure of a transmembrane adhesion or signaling molecule. FCMs, characterized by *sns* expression, move towards the FCs in response to Duf, which act as a myoblast attractant [8]. There are also a number of genes that are differentially expressed in FCs and FCMs [9,10]. For example, *rolling pebbles* (*rols*) and *antisocial* (*ants*), genes whose products are essential for fusion, are expressed only in the FCs [9]. On the other hand, *lame duck* (*lmd*), *delilah* (*del*), and *hibris* (*hbs*) are expressed only in FCMs [9]. Lmd, a transcription factor, acts as a key regulator of myogenesis by

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controlling fusion-competent myoblast development, for example, *myosin heavy chain* expression in FCMs is dependent on lmd [11]. FCMs are also enriched in transcripts of *blown fuse* (*blow*) and *d-titin* in comparison to FCs [9]. Blow and D-titin are required for proper myoblast fusion in *Drosophila* [10].

In *Drosophila* Schneider line 2 cells, ectopic expression of *daughterless*, the *Drosophila* homologue of vertebrate E-protein, resulted in myogenic differentiation, and the conversion efficiency increased from 0.7% to 2.5% upon co-expression of *dmef2*, a MADS-box transcription factor, and *nautilus*, the sole MyoD homologue in *Drosophila* [12]. These results indicate that Schneider cells were derived from the mesoderm of *Drosophila* embryo.

In response to DNA double-strand breaks (DSBs), proliferating eukaryotic cells terminate cell division cycle through checkpoints that monitor DNA integrity [13]. These checkpoints are conserved from yeast to human highlighting the prime importance of DNA repair. Cells resume proliferation once the damage is repaired. If the damage is extensive that is beyond repair, cells undergo apoptosis. DNA DSBs are nevertheless sometimes generated deliberately and for a defined biologic purpose, for example, V(D)J recombination in developing B- and T-lymphocytes which form the basis for the immune diversity [14]. DNA DSBs have been implicated in various differentiation processes, for example, in the budding yeast, *Saccharomyces cerevisiae*, DNA damage induces mating-type switching [15]. In vitro, cultured mammalian cells undergo differentiation upon treatment with various DNA DSB-inducing drugs [16–18]. Two DNA damage-associated genes are induced during osteoblast differentiation in vitro [19]. These results indicate that under certain circumstances, there are overlapping between apoptosis and differentiation with respect to the components of the signaling pathways.

Cells experience replication stress due to inhibition of the elongation step of DNA replication [20]. Replication fork movement can be blocked by inhibitors of DNA polymerase-α, for example, aphidicolin [21], or by inhibitors of ribonucleotide reductase to deplete dNTPs, for example, hydroxyurea (HU) [22]. Replication stress-inducing agents have been implicated in the differentiation of cultured mammalian cells [23,24]. Replication stress can also be induced due to the pyrimidine-adduct formation by UV or base-alkylation by alkylating agents, e.g., ethylmethanesulfonate (EMS) [20].

We previously reported that low doses of DNA DSB-inducing drugs induce myogenic differentiation of Schneider cells with conversion efficiency of ~20% [25]. In this study, we showed that Schneider cells myogenically differentiate upon introduction of DNA DSBs. We also found that HU, aphidicolin, and EMS, drugs that cause replication stress, induced myogenic differentiation of Schneider cells with conversion efficiencies of 15–30%. These systems provide convenient ways to study *Drosophila* myogenesis in vitro.

2. Materials and methods

2.1. Materials

ICRF-193 (Zenyaku Kogyo Co. Ltd., Tokyo, Japan), and aphidicolin (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) were dissolved in DMSO (Wako Pure Chemicals, Osaka, Japan). Adriamycin, hydroxyurea (dissolved in distilled water), etoposide, okadaic acid (all dissolved in DMSO), and lovastatin (dissolved in absolute ethanol) were from Wako. Neocarzinostatin (Yamanouchi Pharmaceutical Co. Ltd., Tokyo, Japan) was dissolved in distilled water. Mitoxantrone (dissolved in absolute ethanol), wortmannin, SB 203580, H-89, KN-62 (all dissolved in DMSO), lactacystin, MTT (both dissolved in distilled water), and EMS were from Sigma (St. Louis, MO, USA). PD 098059, U 0126, AG 490, and nocodazole (all dissolved in DMSO) were from Calbiochem (Darmstadt, Germany). Methylene blue (dissolved in distilled water) was from Kanto Chemical Co. (Tokyo, Japan). SP 600125 (dissolved in DMSO) was from Biomol (Plymouth meeting, PA, USA). Fetal bovine serum (FBS) was from Equitech-Bio Inc. (Kerrville, TX, USA). Phosphate-buffered saline without $MgCl_2$ and $CaCl_2$ [PBS(−)], distilled water and penicillin–streptomycin were from Invitrogen (Carlsbad, CA, USA). 4′,6-Diamidino-2-phenylindole (DAPI) was from Sigma. All other chemicals were reagent grade.

2.2. Cell culture

A clonal stock of *Drosophila* Schneider line 2 cells [26,27] prepared by limited dilution [28] was cultured at 27 °C in Schneider's Drosophila medium (Invitrogen) supplemented with 10% heat-inactivated FBS, 0.5% (w/v) polypeptone (Nihon Seiyaku, Tokyo, Japan), and 1% penicillin–streptomycin. Cells were routinely cultured in tightly capped, 75-cm^2 tissue culture flasks (Iwaki) and the attached cells were passaged in every 3–4 days at 1:5 dilution in fresh medium after gentle scraping with cell scraper (Falcon).

2.3. γ-Irradiation of Drosophila Schneider cells

Exponentially proliferating Schneider cells were plated onto 60-mm tissue culture dishes (Iwaki) and incubated overnight. On the next day, cells were irradiated at room temperature (25 °C) with increasing doses of γ-ray using a ^{137}Cs source. Cells were then incubated at 27 °C and the number of cells with elongated morphology was counted microscopically after 24 h.

2.4. DNA topoisomerase II inhibitor treatment of Schneider cells

Exponentially proliferating Schneider cells were plated onto 24-well plates for 12 h. Cells were then treated with

0.2 μM etoposide or 10 μM ICRF-193 or 0.2 μM etoposide together with 10 μM ICRF-193. In case of pretreatment, ICRF-193 was added 2 h before etoposide. For posttreatment, etoposide was added first and ICRF-193 was added after 2 h. Similarly, co-, pre- or posttreatment of cells with ICRF-193 was performed with adriamycin, mitoxantrone, or NCS. The number of cells with elongated morphology was counted microscopically after 24 h of drug treatment.

2.5. Treatment of Schneider cells with DNA replication inhibitors

Exponentially proliferating Schneider cells were plated onto 24-well tissue culture plates (Iwaki) for 12 h followed by treatment with increasing concentrations of HU, aphidicolin, or EMS. Cells with elongated morphology were counted under microscope at the indicated times. Cell viability was measured by MTT assay as described previously [25]. Briefly, 100 μl of freshly prepared MTT (2.5 mg/ml in PBS) was added into each well of 24-well plate followed by incubation for 4 h at 27 °C. Cells were lysed with 500-μl lysis buffer (11% SDS, 50% v/v 2-propanol) and incubated for 3 h with shaking at room temperature. The reaction products were measured at A_{570} and corrected against blank (blank is the media processed in absence of cells). Relative viability was calculated as follows: Relative viability=$(A_e/A_c)\times 100$ where A_e is the absorbance of drug-treated cells (experimental) and A_c is the absorbance of solvent-treated cells (control).

2.6. Myosin heavy chain immunostaining

Myosin heavy chain immunostaining was performed as described previously [25]. Schneider cells were plated onto poly-L-lysine-coated cover slips and incubated at 27 °C for 12 h followed by 24-h treatment with drugs or γ-ray. After drug treatment, cells were washed with PBS(−), fixed with methanol for 15 min at −20 °C followed by permeabilization with 0.2% Triton X-100 in PBS(−) for 5 min at 27 °C. Permeabilized cells were washed with PBS(−) followed by incubation in blocking buffer (3% BSA in PBS) for 30 min. Cells were then incubated at 27 °C with anti-myosin heavy chain antibody (1:500) (kindly provided by Dr. Bruce M. Paterson) in blocking buffer for 3 h. DNA was stained after incubating cells with 1 μM DAPI in PBS(−). Immunecomplex with anti-MyHC antibody was detected after 1-h incubation at 27 °C with the secondary antibody Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200) (Molecular Probes, Eugene, OR, USA).

2.7. Signaling pathway inhibitor treatment

Schneider cells at a density of 1×10^4 were plated in each well of 24-well plates followed by treatment with various inhibitors of signaling pathways for 1 h. Cells were then treated with 2 mM HU and the number of cells with elongated morphology was counted microscopically after 24 h.

2.8. Proteasomal inhibitor treatment

Lactacystin, a proteasomal inhibitor, was added to exponentially proliferating Schneider cells at a concentration of 0.15 μM for 1 h followed by treatment with 2 mM HU. The number of cells with elongated morphology was counted microscopically after 24 h.

2.9. RT-PCR analyses of myogenic gene expression

Exponentially proliferating Schneider cells were treated with 20 nM NCS or 2 mM HU followed by total RNA isolation at 0, 12, and 24 h. Total RNA was isolated by using the TRIzol reagent of Invitrogen according to the manufacturer's instructions. For RT-PCR analysis, cDNAs were synthesized from total RNA using the reverse transcriptase kit of Stratagene (La Jolla, CA, USA) with oligo-dT primer according to the manufacturer's instructions. The PCR reaction mixture consisted of 125-ng cDNA, 200 μM of each of dNTPs, 1.25 mM $MgCl_2$, 50 pmol of each of the primers, and 1.25 U AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA, USA) in a reaction volume of 50 μl. The PCR amplification cycles consist of 35 or 40 cycles of denaturing at 94 °C for 1 min, annealing at 44 °C for 1 min and extension at 72 °C for 2 min, followed by a final extension at 72 °C for 7 min. *rp49* was used as an internal control in all the PCR amplification cycles. The expected PCR product size was between 700 and 800 bp for *lmd*, *sns*, *del*, *blow*, *d-titin*, *duf*, *ants*, and *rols*. For *rp49*, the PCR product size was 405 bp. The PCR products were resolved by 1.5% agarose gel electrophoresis and stained with ethidium bromide. The identities of PCR products were confirmed by restriction digestion analyses (data not shown). The sequences of other primers used are as follows: *lmd*, forward: 5′-GGAAACGAATGAAAGTTACG-3′, reverse: 5′-TGAGCCATATTGTTGTGGTA-3′; *sns*, forward: 5′-AGAAGCGAGTGCTAGATTTG-3′, reverse: 5′-AGTCAAAGATACGGATGTGG-3′; *del*, forward: 5′-ACCAAAATCACCACCCTGAG-3′, reverse: 5′-ATATCCGGAAAAATCGCTGTG-3′; *blow*, forward: 5′-GAGAAAAGCAACAGCCCAAG-3′, reverse: 5′-CTGAACATGGTGGACAGGTG-3′; *d-titin*, forward: 5′-CAAGCATGGAACTGCTGAAA-3′, reverse: 5′-CGACTTTGAGGCACACTTGA-3′; *duf*, forward: 5′-TTAGCGCCATCTATGGTAAT-3′, reverse: 5′-AACGAAAGCAGTCACATTCT-3′; *ants*, forward: 5′-CTTCGACAAGGGCAAGAGAC-3′, reverse: 5′-ATGCTGTGTCAATGGGACAA-3′; *rols*, forward: 5′-GCCACATTGGATTATCAGTGA-3′, reverse: 5′-CATGATGTCATTCCGATTGAAG-3′; *rp49*, forward: 5′-TACAGGCCCAAGATCGTGAA-3′, reverse: 5′-

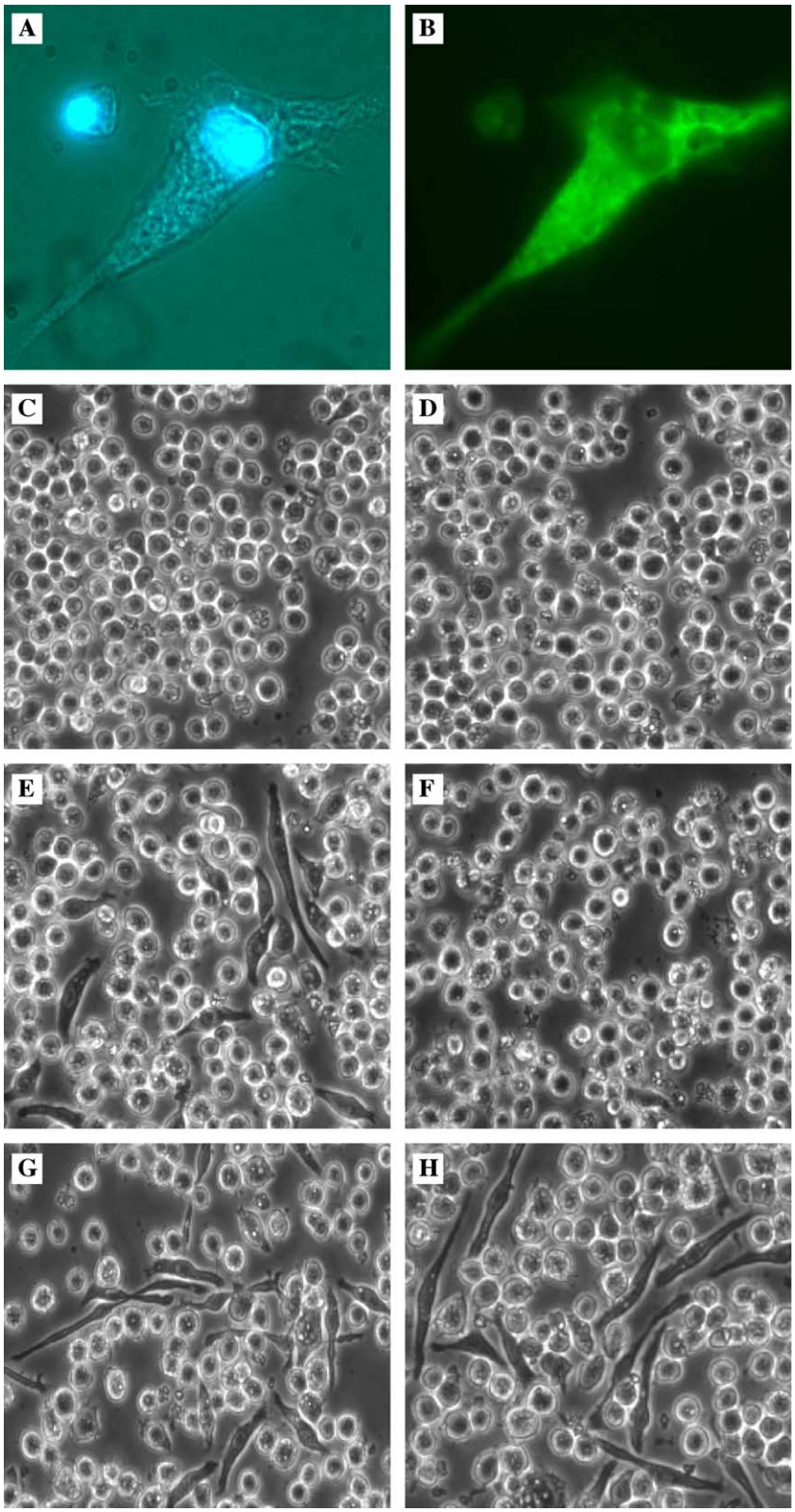

Fig. 1. ICRF-193 blocked etoposide-induced differentiation of *Drosophila* Schneider cells. (A) Phase-contrast and DAPI-staining, and (B) anti-myosin heavy chain staining of etoposide-treated Schneider cells. (C–H) Cells were treated for 24 h with (C) DMSO; (D) 10 μM ICRF-193; (E) 0.2 μM etoposide; (F) 0.2 μM etoposide together with 10 μM ICRF-193; (G) 20 nM NCS; (H) 20 nM NCS together with 10 μM ICRF-193.

ACCGTTGGGGTTGGTGAG-3′. Primers for *dmef2*, *daughterless*, and *nautilus* were described previously [12].

3. Results

3.1. ICRF-193, a catalytic inhibitor of DNA topoisomerase II (topo II), inhibits differentiation of Schneider cells induced by DNA DSB-inducing topo II inhibitors

To understand the molecular mechanisms underlying myogenic differentiation of *Drosophila* Schneider cells by DNA DSB-inducing drugs [25], we examined the influence of two types of topo II inhibitors on the process. The first type of topo II inhibitors are known as "cleavable-complex" stabilizing drugs that cause DNA DSBs, for example, etoposide, adriamycin, mitoxantrone, etc. [29]. The second type of topo II inhibitors are known as "catalytic inhibitors" as they do not introduce DNA DSBs, for example, ICRF-193, merbarone, aclarubicin, etc. [30]. We previously reported DNA DSB-inducing topo II inhibitors—etoposide, adriamycin, and mitoxantrone—induced myogenic differentiation of Schneider cells, but no differentiation was observed with ICRF-193 [25]. In the present study, we examined the influence of ICRF-193 on the differentiation induced by DNA damaging topo II inhibitors. If the differentiation of Schneider cells upon treatment with DNA damaging topo II inhibitors is due to the generation of DNA DSBs, ICRF-193 should block etoposide-induced differentiation. Etoposide-induced converted Schneider cells were myosin heavy chain-positive, whereas the round cells were myosin-negative (Fig. 1A and B). Treatment of cells with ICRF-193 did not induce differentiation (Fig. 1D). When Schneider cells were co-treated with ICRF-193 and etoposide, significant inhibition of etoposide-induced differentiation was observed (Fig. 1E and F). No such inhibitory effect of ICRF-193 was observed on the differentiation induced by NCS, a drug that causes DNA DSBs independent of topo II (Fig. 1G and H). In case of 2-h posttreatment, ICRF-193 did not significantly inhibit etoposide-induced differentiation (Fig. 2). Similar results were observed with two other DNA DSB-inducing topo II inhibitors, adriamycin and mitoxantrone (Fig. 2). These results support the notion that DNA DSBs induce myogenic differentiation of Schneider cells. As reported previously [25], we reproducibly observed differentiated (0.1–0.5%) cells in the absence of drugs even with clonal stocks (data not shown). It is not clear how these cells become differentiated, but the most plausible explanation is inefficient repair of spontaneously generated DNA DSBs in a population of Schneider cells resulting in differentiation. Treatment with DNA damaging drugs or replication inhibitors differentiated two of four Schneider line 2 cells collected from four different sources (data not shown). We do not know why two of the Schneider cell lines were refractory to differentiation, but Schneider cell lines are heterogeneous with respect to responsiveness to wingless signaling [28], which is involved in muscle development in *Drosophila* [31].

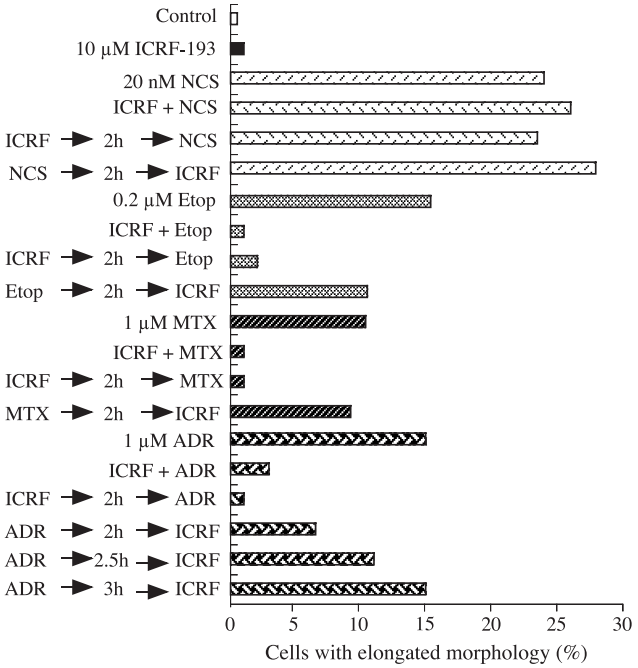

Fig. 2. ICRF-193 attenuates differentiation of *Drosophila* Schneider cells induced by DNA DSB-inducing topo II inhibitors in case of co- or pretreatment but not in the case of 2-h posttreatment. Cells were treated with etoposide (Etop), adriamycin (ADR), mitoxantrone (MTX), or NCS together with ICRF-193 (cotreatment), or 2 h after ICRF-193 addition (pretreatment), or 2–3 h before ICRF-193 addition (posttreatment). The data shown are representatives of three different experiments with similar results.

3.2. γ-Irradiation induces differentiation of Drosophila Schneider cells

To further demonstrate that DNA DSBs can induce differentiation of Schneider cells, we examined whether physical introduction of DNA DSBs by γ-irradiation can induce differentiation of Schneider cells. As shown in Fig. 3A and B, *Drosophila* Schneider cells differentiated upon treatment with γ-ray with the conversion efficiency of ~15%. The converted cells were myosin-positive as indicated by myosin heavy chain staining, whereas round cells were not (Fig. 3C and D) The results suggest that Schneider cells undergo myogenic differentiation upon treatment with γ-ray. Since γ-irradiation causes DNA DSBs, the results support the notion that Schneider cells differentiate in response to DNA DSBs.

3.3. Induction of myogenic differentiation of Schneider cells by DNA replication inhibitors

We previously reported that low concentrations of DNA DSB-inducing drugs, for example, NCS, caused myogenic

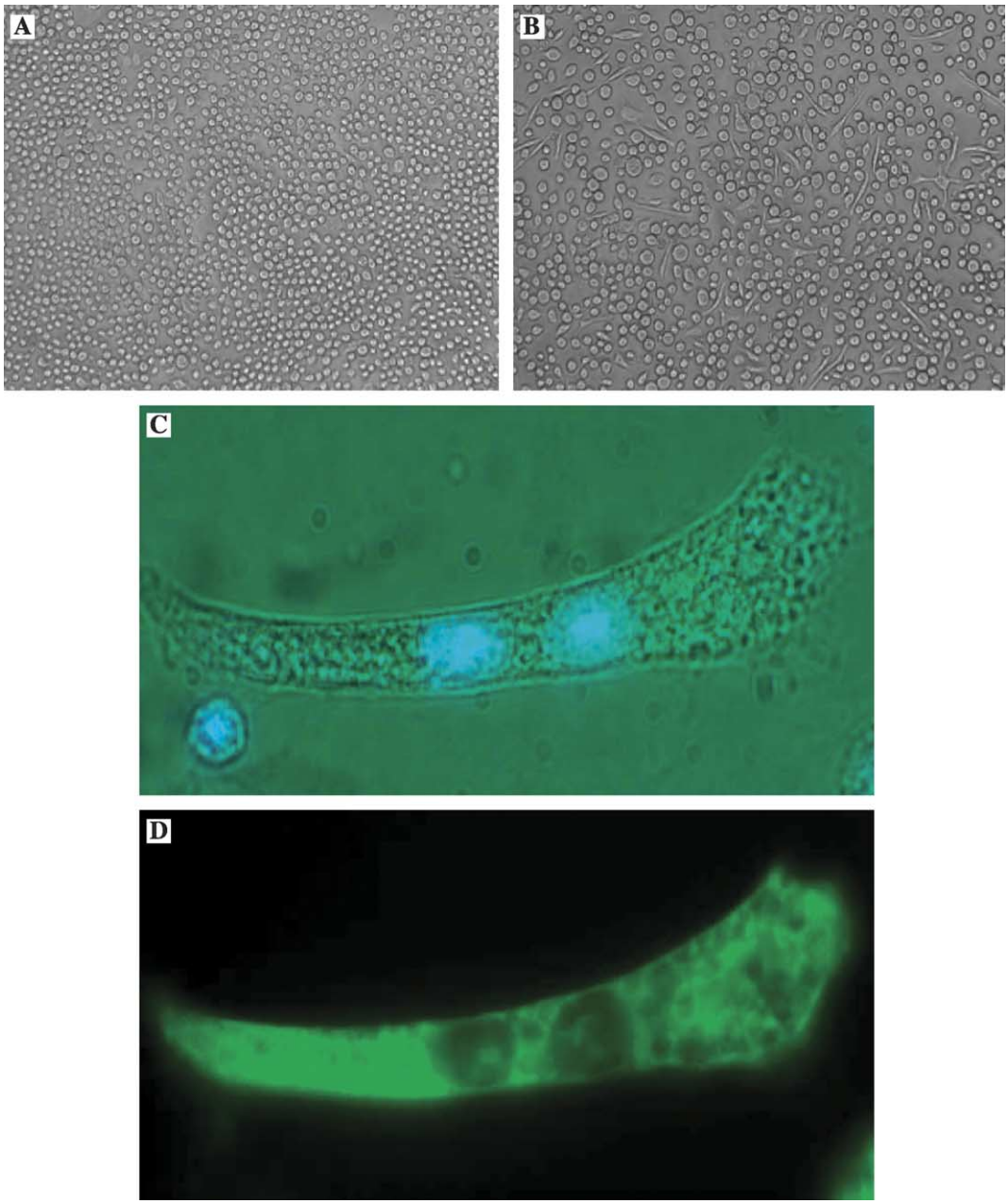

Fig. 3. γ-Irradiation induces myogenic differentiation of *Drosophila* Schneider cells. (A) Untreated control cells; (B) cells irradiated with γ-ray (90 Gy) at a dose rate of 2.5 Gy/min. (C) Phase-contrast and DAPI-staining, and (D) anti-myosin heavy chain staining of γ irradiated Schneider cells. Cells were observed microscopically 24 h after irradiation. The data shown are representative of two different experiments with similar results.

differentiation of Schneider cells with conversion efficiency of ~20% [25]. To increase the percentage of converted cells, we attempted to synchronize cells at a particular phase using various drugs that are known to cause cell cycle arrest. We found that treatment of Schneider cells with DNA replication inhibitors, HU or aphidicolin, resulted in morphological differentiation of 15–30% cells (Fig. 4A–C). Cells with elongated morphology started appearing ~12 h after HU addition and increased until 24 h (data not shown). We also observed differentiation of cells with 5 mM EMS (Fig. 4D). No differentiation was observed upon treatment of cells with nocodazole (20 μM), ICRF-193 (10 μM), rapamycin (5 nM) or lovastatin (400 μM), drugs that arrest cell cycle at the M, G2 or G1 phase, indicating that mere inhibition of cell proliferation does not induce differentiation of Schneider cells (data not shown; Ref. [25]). These results indicate that DNA replication inhibitors induce morphological differentiation of Schneider cells.

To demonstrate that HU-induced converted Schneider cells were indeed myogenic, we performed immunostaining with anti-myosin heavy chain antibody. HU-induced converted cells were myosin-positive but the round-shaped cells were not (Fig. 5A and B). Around 1% of the converted cells

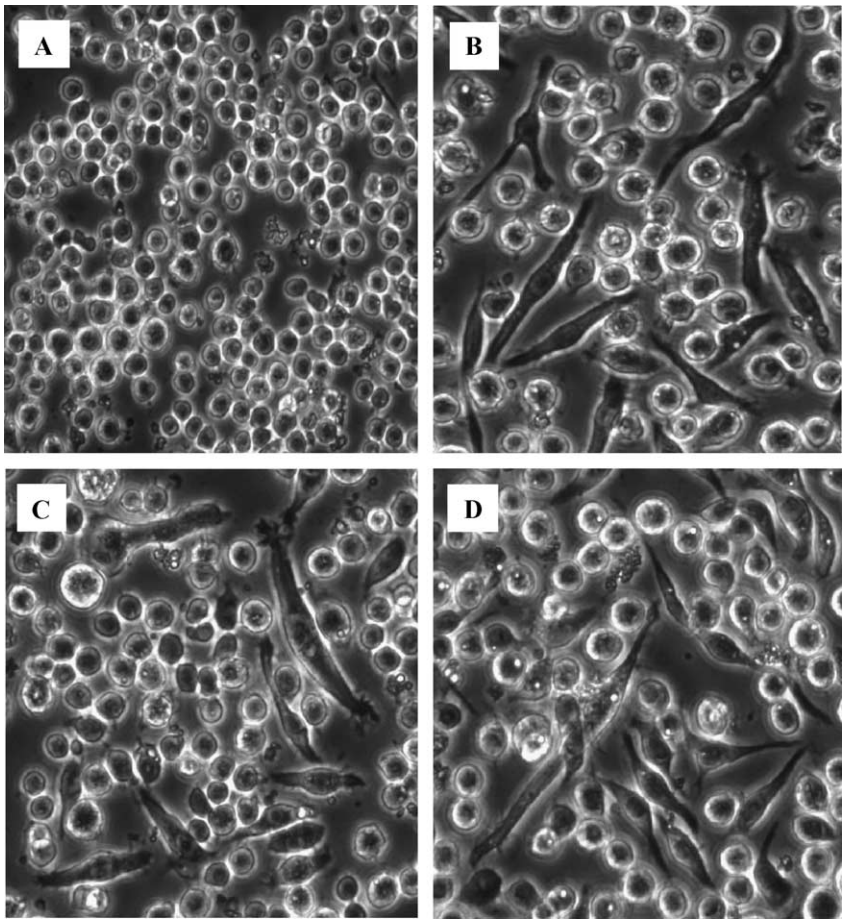

Fig. 4. Replication stress-inducing drugs cause morphological differentiation of *Drosophila* Schneider cells. Cells were treated for 24 h with (A) DMSO; (B) 2 mM HU; (C) 25 μM aphidicolin; or (D) 5 mM EMS. Cell morphology was observed microscopically after 24-h treatment.

showed multinucleation as determined by DAPI-staining (Fig. 5C). Myosin heavy chain immunostaining indicated that aphidicolin- and EMS-induced converted Schneider cells were also myosin-positive (data not shown). The results indicate that DNA replication inhibitors induce myogenic conversion of *Drosophila* Schneider cells.

3.4. HU-induced differentiation is dependent on PP 1/2A, p38 MAPK and JNK signaling pathways

We previously reported that NCS-induced myogenic differentiation of Schneider cells is dependent on PP 1/2A, p38 MAPK, and JNK [25]. In the present study, we examined the influence of specific inhibitors of various signaling pathways on the HU-induced differentiation of Schneider cells. As shown in Fig. 6, pretreatment of Schneider cells with okadaic acid, SB 203580, and SP 600125—inhibitors of protein phosphatase 1/2A (PP 1/2A), p38 MAPK, and JNK—resulted in significant inhibition of HU-induced morphological changes of Schneider cells. There was no significant effect on cell viability of the inhibitors at the concentrations used with or without HU (data not shown). The concentrations of inhibitors were chosen after titration at the range of 0–100 μM. No effect was apparent on the morphology or number of HU-induced differentiated cells upon pretreatment with up to 100 μM each of wortmannin, methylene blue, PD 098059 or U 0126, AG 490, KN-62, H-89, which are inhibitors of PI-3 kinase, NO synthase, MAPKK, JAK, Ca^{2+}-calmodulin-dependent PK, PKs-A and-G, respectively (data not shown). The results indicate that HU-induced myogenic conversion of Schneider cells is dependent on PP 1/2A, p38 MAPK, and JNK.

3.5. HU-induced differentiation is blocked by proteasomal inhibitor, lactacystin

Proteasomal activity is essential for the induction of murine myogenesis [32]. We previously reported that NCS-induced differentiation of Schneider cells is dependent on proteasomes [25]. In the present study, we examined whether proteasomal activity is also required for the HU-induced differentiation of *Drosophila* Schneider cells. We found that pretreatment of Schneider cells with lactacystin, a highly specific proteasomal inhibitor, resulted in a significant inhibition of HU-induced differentiation (Fig. 7). No effect of 0.15 μM lactacystin on cell viability was observed with or without HU after 24-h treatment (data not shown).

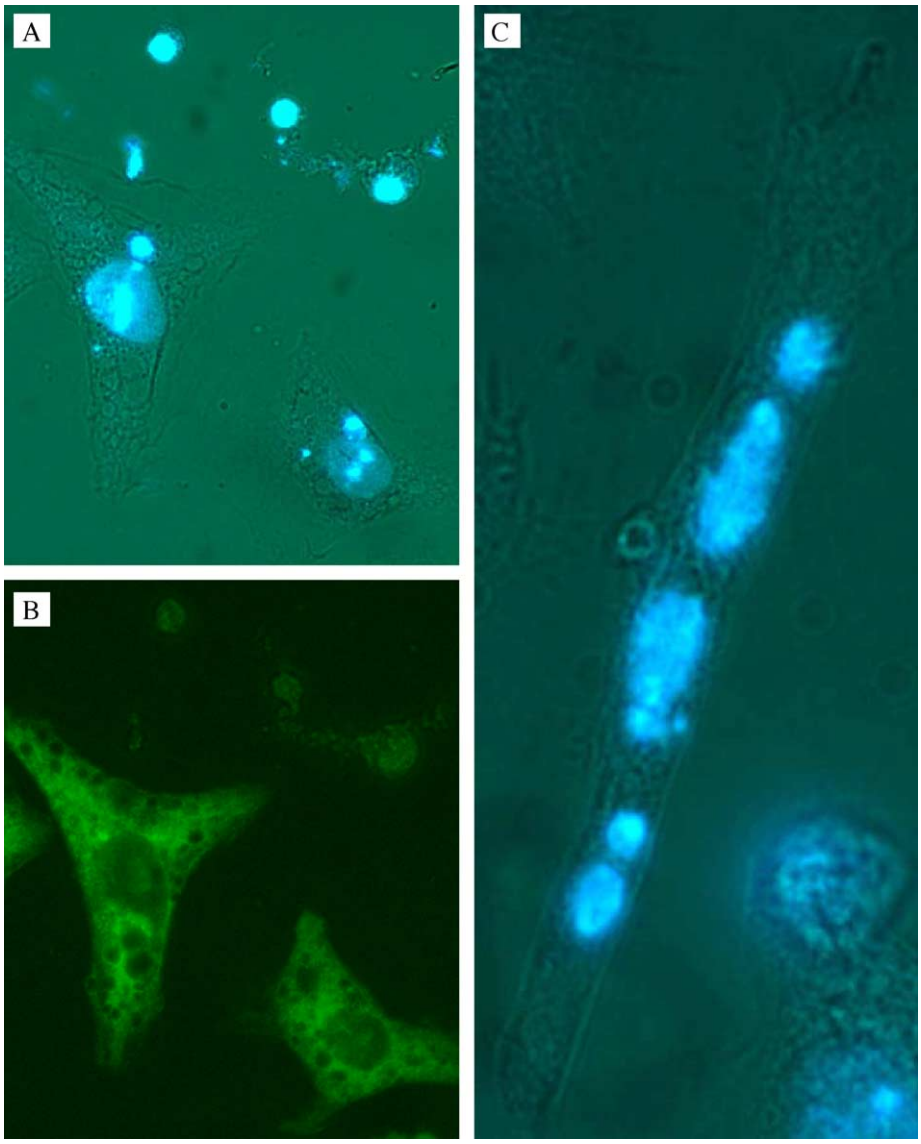

Fig. 5. HU-induced converted *Drosophila* Schneider cells are myosin heavy chain-positive and multinucleated. (A) Phase-contrast and DAPI-staining of round and elongated Schneider cells after 24-h treatment with 2 mM HU; (B) elongated cells were stained positive for myosin heavy chain; (C) phase-contrast and DAPI-staining of multinucleated, elongated Schneider cells observed upon HU treatment.

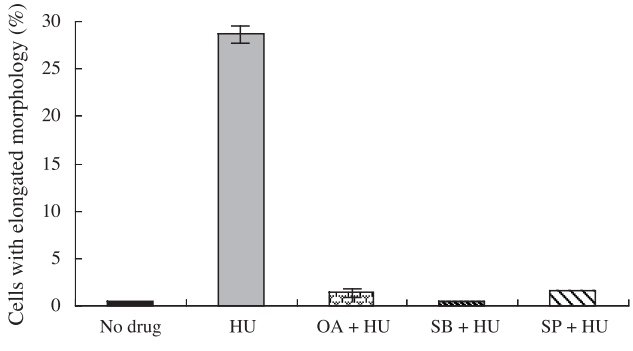

Fig. 6. Inhibition of HU-induced morphological differentiation of *Drosophila* Schneider cells by signaling pathway inhibitors. Cells were pretreated for 1 h with 4 nM okadaic acid (OA); 40 μM SB 203580 (SB) or with 40 μM SP 600125 (SP) followed by treatment with 2 mM HU for 24 h. Cells with elongated morphology were counted under microscope. The data shown are averages of two independent experiments with similar results.

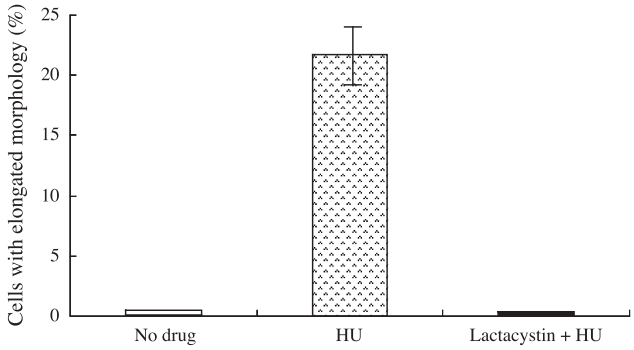

Fig. 7. Lactacystin, a proteasomal inhibitor, inhibits HU-induced differentiation of Schneider cells. Cells were pretreated with 0.15 μM lactacystin for 1 h followed by 24-h treatment with 2 mM HU. The data shown are averages of two independent experiments with similar results.

The results indicate that proteasomal activity is required for the myogenic differentiation of Schneider cells by HU.

3.6. Induction of fusion-competent myoblast-specific gene expression in Schneider cells upon treatment with NCS or HU

To further elucidate the molecular mechanisms underlying myogenic conversion of Schneider cells, we examined the expression of myogenic genes in Schneider cells treated with NCS or HU. RT-PCR analyses revealed that the expression of FCM-specific myogenic genes, *lmd*, *sns*, and *del*, were significantly induced after 24 h in NCS- or HU-treated Schneider cells (Fig. 8A). *blow* and *d-titin*, genes that are preferentially expressed in FCMs [9], were also induced by both NCS and HU within 12 h of treatment (Fig. 8A). Under our experimental conditions, we did not detect expression of the FC-specific gene *duf* in Schneider cells after drug treatment, although expression was detected in *Drosophila* embryos (stage 0–16 h) under the same PCR conditions (Fig. 8B). Similarly, we detected no *rols* or *ants* expression, although their expression was detected when embryonic RNA was used (data not shown). Consistent with the previous report [12], the present study demonstrated

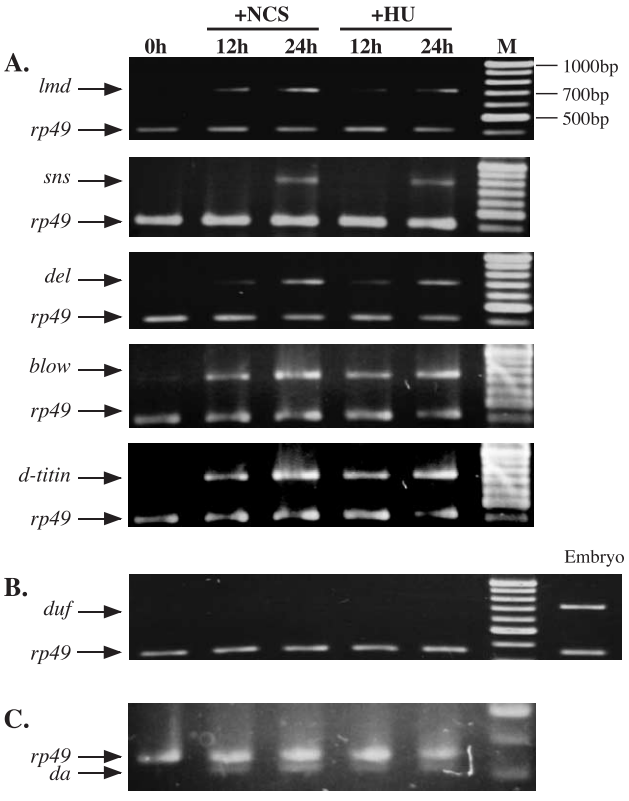

Fig. 8. Induction of fusion-competent myoblast-specific gene expression in *Drosophila* Schneider cells by NCS or HU. RT-PCR analyses of: (A) FCM-specific genes—*lmd*, *sns*, *del*; and FCM-enriched genes—*blow*, *d-titin*; (B) FC-specific gene—*duf*; and (C) myogenic gene—*da*. *rp49* was used as an internal control. The PCR cycle number was 35 except for *sns* and *duf* [40]. The data shown are representative of two different experiments with similar results.

dmef2 and *nautilus* (*nau*) expression in Schneider cells under standard conditions, and expression was not significantly induced by treatment with NCS or HU (data not shown). On the other hand, the expression of the myogenic gene *da* was induced by NCS or HU within 12 h of treatment (Fig. 8C). NCS or HU did not affect expression of the ribosomal protein, *rp49*, which served as an internal control in all samples (Fig. 8A–C). These results indicate that NCS or HU significantly induced expressions of FCM-specific genes in *Drosophila* Schneider cells.

4. Discussion

In the present study, the expression of fusion-competent myoblast-specific genes was induced during myogenic differentiation of *Drosophila* Schneider cells by DNA DSBs or replication inhibition. The systems described in this study provide convenient ways to study *Drosophila* myogenesis in vitro.

We previously reported that DNA DSB-inducing drugs induce myogenic differentiation of *Drosophila* Schneider cells [25]. In this study, we examined the molecular mechanisms underlying the process. We demonstrated that replication stress-inducing drugs caused myogenic differentiation of Schneider cells (Figs. 4, 5). It remains to be shown how DNA DSBs and replication stress initiate differentiation of Schneider cells. One possible explanation is that S phase arrest induces myogenic program in Schneider cells, that is, cells in S phase undergo myogenic differentiation upon treatment with DNA DSB- or replication stress-inducing drugs [14,21]. This hypothesis is supported by the following findings: (i) the percentage of converted cells by either NCS or HU was 20–30%, which is close to the percentage of S phase cells in a random culture of Schneider cells (the duration of S phase in Schneider cells is ~6 h and the cell division cycle is ~20 h in our culture conditions); and (ii) the same cellular components are apparently required for NCS- and HU-induced differentiation processes (Figs. 6 and 7; Ref. [25]). In *Drosophila*, mei-41, the homologue of ATM/ATR kinase, is essential for the replication-arrest during the 13th cell cycle of the embryo [33]. Replication stress during the 13th cell cycle occurs most probably due to the depletion of maternally provided replication factors, and the role of mei-41 is to prevent premature entry into M phase by delaying S phase so that zygotic gene expression can ensue [33]. It is interesting to note that mei-41 is activated in response to both DNA DSBs and replication stress [34]. It can be speculated that both NCS and HU stimulate mei-41-mediated signaling which, in turn, activates the myogenic program in Schneider cells. Another explanation for myogenic differentiation by DNA damaging drugs is that, in the presence of NCS or HU, the persistence of less condensed, that is, more unfolded, S phase chromatin allows the expression of myogenic genes in Schneider cells. We

cannot exclude the possibility that replication stress-inducing drugs are also causing DNA DSBs. However, this possibility seems unlikely as both HU and aphidicolin are widely used to synchronize cells at S phase. If HU or aphidicolin induces significant DNA damage, cells would have been arrested at different phases of cell cycle as observed with DNA DSB-inducing drugs [35]. Puri et al. [36] reported inhibition of myogenic differentiation of mouse C2C12 cultured cells in response to DNA damage. The authors proposed the presence of a DNA damage-checkpoint that prevents differentiation of muscle precursor cells. It is possible that the nature of DNA damage-checkpoint in mouse C2C12 cells is different from that of *Drosophila* Schneider cells, and therefore Schneider cells undergo differentiation in response to DNA damage.

To identify the signaling pathways that might be required for the HU-induced differentiation, we treated Schneider cells with specific chemical inhibitors. We showed that HU-induced differentiation is dependent on PP 1/2A, p38 MAPK, and JNK (Fig. 6). PP 1/2A and p38 MAPK are required for the myogenesis in mammals [37,38]. It is possible that the roles of PP 1/2A and p38 MAPK during myogenic differentiation have been evolutionarily conserved. Activation of JNK inhibits mammalian myogenesis [39]. Our data suggest that JNK is required for the proper myogenesis in *Drosophila* Schneider cells (Fig. 6). It is possible that JNK plays unique role(s) during *Drosophila* myogenesis. It would be worthwhile to determine the precise functions of the signaling pathway components during drug-induced differentiation of Schneider cells, so that the findings can be extended to *Drosophila* myogenesis in vivo.

Proteasomal activity is essential for myogenesis in mammals [40]. Degradation of cyclin D1 by proteasomes is required for the initiation of myogenesis [41]. In the present study, we showed that HU-induced differentiation of Schneider cells was inhibited by proteasomal inhibitor, lactacystin (Fig. 7). It is possible that the persistence of proteins required during the mitotic cell cycle might prevent myogenic differentiation of *Drosophila* Schneider cells by HU. It would be worthwhile to identify the proteins that undergo proteasomal degradation during the myogenic differentiation of Schneider cells by HU.

In this study, we also found that the expression of myogenic genes *lmd*, *sns*, and *del* was induced in *Drosophila* Schneider cells upon treatment with NCS or HU (Fig. 8A). We also detected significant induction of expression of two other myogenic genes, *blow* and *d-titin* (Fig. 8A). In vivo, *lmd*, *sns*, and *del* are expressed in FCMs with no detectable expression in FCs [9]. FCMs are also enriched with the transcripts of *blow* and *d-titin* [9]. We failed to detect any induction of expression of founder cell (FC)-specific gene-*duf* (Fig. 8B). We also failed to detect any expressions of two other founder cell-specific genes, *rols* and *ants*, in Schneider cells after drug treatment (data not shown). The lack of expression of *duf*, *rols*, and *ants* provides a possible explanation for the observed lack of fusion during drug-induced differentiation of Schneider cells because all these genes are essential for myoblast fusion in vivo. In a previous report, Wei et al. [12] reported myogenic conversion of Schneider cells by overexpression of *daughterless*, and observed more multinucleated cells than that we observed in our system. It is possible that the genes involved in the fusion process are weakly induced by DNA damage or replication inhibition. It would be worthwhile to determine whether overexpression of FC-specific genes like *duf*, *rols*, and/or *ants* can increase the number of multinucleated myoblasts during drug-induced differentiation of Schneider cells.

ICRF-193, a non-DNA-damaging topo II inhibitor, blocked differentiation induced by DNA DSB-inducing topo II inhibitors—etoposide, adriamycin, and mitoxantrone (Figs. 1 and 2). DNA DSB-inducing topo II inhibitors are powerful anticancer agents [42]. The in vitro differentiation system based on Schneider cells described in this study can be used to perform preliminary screening of compounds that can reduce the cytotoxicity of anticancer drugs in vivo. For example, ICRF-193 reduced cytotoxicity induced by etoposide or adriamycin in mammalian cells both in vitro and in vivo [43–45]. The advantages of our in vitro differentiation system are that (1) it is extremely easy to perform, and (2) it is very rapid and cost-effective.

In summary, we have shown that DNA DSBs or replication inhibition induces myogenic differentiation of *Drosophila* Schneider cells. We have also described some of the cellular components that are apparently required for proper myogenesis. It would be worthwhile to perform genome wide gene expression analysis to identify novel gene(s) that is(are) required for the drug-induced differentiation of Schneider cells. Identification and characterization of such novel genes would allow us to study their roles during *Drosophila* muscle formation in vivo.

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